

## Research Note

Improved methods for separation of wheat endosperm proteins  
and analysis by two-dimensional gel electrophoresis

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Fractionation of seed proteins has been a significant area of interest and activity, since Osborne (1924) introduced his solubility fractionation methods. In particular, the fractionation of wheat endosperm proteins has presented numerous challenges, because of their unique solubilities. Recently, we combined solubility fractionation and 2-DE protein analysis methods that taken separately may not seem unusual, but together resulted in an approach enabling us to identify a number of thioredoxin target proteins in wheat endosperm (Wong et al., 2003, 2004). Proteins are recovered from the various fractions by precipitation with methanol or acetone. A finding of interest to cereal scientists is that SDS can be used to solubilize the gluten proteins and, following acetone precipitation, the recovered proteins can be further characterized by IEF, HPLC, and mass spectroscopy.

Wheat (*Triticum aestivum* L. 'Butte 86') was grown in a climate-controlled greenhouse as described previously (Wong et al., 2003). Mature grain was tempered and milled to flour with a Brabender Quadrumat Junior (South Hackensack, NJ) using standard procedures at the Western

Wheat Quality Laboratory (US Department of Agriculture, Agricultural Research Service, Pullman, WA). Flour proteins were separated into the gluten, metabolic, and chloroform/methanol-soluble (CM) proteins fractions based on solubility in KCl and methanol (Fig. 1). Flour (50mg) was suspended in 200 µl of cold (4 °C) KCl buffer (50 mM Tris-HCl, 100 mM KCl, 5 mM EDTA, pH 7.8; Bean and Lockhart, 1998). The suspension was incubated on ice for 5 min with intermittent mixing and centrifuged at 14,500g for 15 min at 4 °C (Tomy MRX-151; Peninsula Laboratories, Inc., Belmont, CA, USA). The pellet or KCl-insoluble fraction was suspended in 800 µl of SDS buffer (2% SDS, 10% glycerol, 50 mM DTT, 40 mM Tris-Cl, pH 6.8), incubated for 1 h at room temperature, and insoluble material removed by centrifugation at 16,000g for 10 min at room temperature (Eppendorf 5415C; Brinkman Instruments, Inc., Westbury, NY, USA). The proteins were precipitated from the SDS buffer by the addition of 4 vol of cold (−20 °C) acetone and incubation overnight at −20 °C. Following centrifugation, the pellet was rinsed by pipetting cold acetone onto the pellet, centrifuging at 16,000g for 10 min at room temperature, and pipetting the acetone off of the pellet. The pellet (gluten proteins) was dried by vacuum centrifugation (Speed Vac DNA 110; Savant Instruments, Inc., Farmingdale, NY) and solubilized in urea buffer (9 M urea, 4% NP-40, 1% DTT, and 2% ampholytes) to a final concentration of 3 µg protein/µl. The KCl-soluble fraction was collected and 5 vol of 0.1 M ammonium acetate in methanol was added at room temperature. Following incubation overnight at −20 °C, the methanol-insoluble fraction was pelleted by

**Abbreviations:** 2-DE, two-dimensional polyacrylamide gel electrophoresis; CM proteins, salt/chloroform/methanol-soluble proteins; HMM-GS, high molecular mass-glutenin subunits; IEF, isoelectric focusing; LMM-GS, low molecular mass-glutenin subunits.

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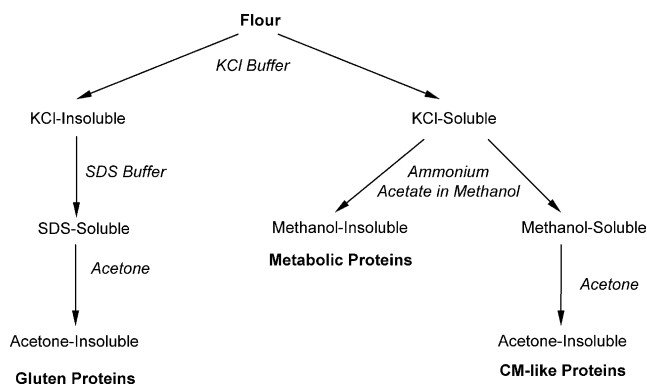


Fig. 1. Separation of wheat flour proteins into gluten, metabolic, and CM-like protein fractions.

centrifugation at 14,500g for 15 min at 4 °C. The pellet (metabolic proteins) was rinsed with cold acetone and solubilized in urea buffer. The proteins in the methanol-soluble fraction were precipitated with acetone, and the pellet (CM-like proteins) was rinsed, and solubilized in urea buffer. For protein determination, triplicate samples were removed from the SDS solubilized KCl-insoluble fraction (5  $\mu$ l), the KCl-soluble fraction (10  $\mu$ l), and the KCl-soluble/methanol soluble fraction (25  $\mu$ l) for protein analysis. Following precipitation of the fractions with the appropriate solvent, protein was quantified by the procedure of Lowry et al. (1951).

Reproducible, high-resolution 2-D gels are not only dependent on sample preparation and solubilization, but also on a multi-step gel electrophoresis protocol. The following combination of techniques has worked well in our hands. We used a mini 2-DE system, because it allows relatively rapid separation of samples limited by protein amount. Proteins were separated in the first dimension by IEF (Mini Protean II Tube Cell; BioRad Laboratories, Richmond, CA) according to the instruction manual with the modifications outlined here. The gels contained 9.2 M urea, 4% (total monomer) acrylamide: Bis, 2% Nonidet P-40, 2% 3–10 Iso-Dalt Grade Servalyts (Crescent Chemical Co., Islandia, NY), 0.015% ammonium persulfate and 0.125% TEMED. The upper electrode (anode) buffer was 0.2% (v/v) sulfuric acid and the lower electrode buffer (cathode) was 0.5% (v/v) ethanolamine. Because the anode buffer was acidic, the wires from the electrophoresis cell were reversed at the power supply. The gels were prefocused at 200 V for 10 min, 300 V for 15 min and 400 V for 15 min. Samples containing 18  $\mu$ g of protein were loaded at the acidic end of the IEF gel and overlaid with 5 M urea. For protein pI determinations, 3  $\mu$ l of 2D SDS-PAGE Standards (BioRad) was added to the sample. IEF gels were run at 500 V for 10 min and then at 750 V for 1 h. Gels were extruded into microcentrifuge tubes using a tube gel ejector attached to a 10 ml syringe without buffer.

Equilibration buffer was added (2.3% SDS, 10% glycerol, 0.05% dithiothreitol, 62.5 mM Tris–Cl pH 6.8) and gels were frozen immediately by placing the tubes in dry ice; tubes were stored at –70 °C. Proteins were separated in the second dimension by SDS gel electrophoresis (XCell SureLock Mini-Cell electrophoresis system; Invitrogen Corp., Carlsbad, CA). IEF gels were thawed, placed on top of Novex NuPAGE Bis–Tris 4–12% acrylamide 1 mm thick gels with 2-D well (Invitrogen Corp.), and overlain with 45  $\mu$ l of equilibration buffer. Molecular weight markers (Mark 12 Unstained Standard; Invitrogen Corp.) were loaded (3  $\mu$ l) into the 2-D well. The SDS gels were run with NuPAGE MES SDS running buffer (Invitrogen Corp.) for 48 min at 200 V. The 2-D gels were stained with Coomassie G-250 (Sigma, St. Louis, MO) by the method of Kasarda et al. (1998), destained in water for 3–5 h, and stored at 4 °C in 20% ammonium sulfate. The stained gels were digitized with a calibrated scanner (PowerLook III, UMAX Technologies, Inc., Dallas, TX) at 300 dpi with the same settings for all gels and the number of protein spots per gel determined by computer analysis (Progenesis Workstation ver. 2004, Non-Linear Dynamics Limited, Newcastle upon Tyne, UK).

The KCl-insoluble, gluten protein fraction contained 84.0% of the total flour protein extracted; 2-DE resolved 478 proteins (Fig. 2A). The pattern was similar to that of the map developed by Payne et al. (1985) and, accordingly, contained four major protein regions. Region 1 corresponds to the high molecular mass glutenin subunits (HMM-GS), region 2 to the  $\omega$ -gliadins (DuPont et al., 2000), region 3 to the  $\alpha$ - and  $\gamma$ -gliadins and low molecular mass glutenin subunits (LMM-GS), and region 4 to the albumins and globulins. We noted one problem with the gluten protein fraction: the starch present in the fraction swelled as it absorbed the urea solubilization buffer, making protein recovery difficult. Consequently, proteins were first solubilized with SDS buffer so that the starch, which remained insoluble, could be removed in the subsequent centrifugation step. Protein preparations containing high concentrations of SDS could not, however, be separated by IEF, because of their high negative charge. This problem was solved by first precipitating and rinsing the proteins with acetone to remove the SDS. Methanol precipitation also removed the SDS, but protein recovery was lower than with acetone precipitation (data not shown). The recovered proteins could then be solubilized with the urea buffer and separated by IEF. The similarity of our gel pattern with that of Payne et al. (1985) confirms the utility of this approach.

Classically, the albumins and globulins are recovered in the KCl solution. The KCl-soluble/methanol-insoluble fraction contained 11% of total flour protein extracted; 2-DE resolved 474 proteins (Fig. 2B). Until recently, very few of these proteins had been identified (Singh et al., 2001; Skylas et al., 2000; Wong et al., 2003, 2004).

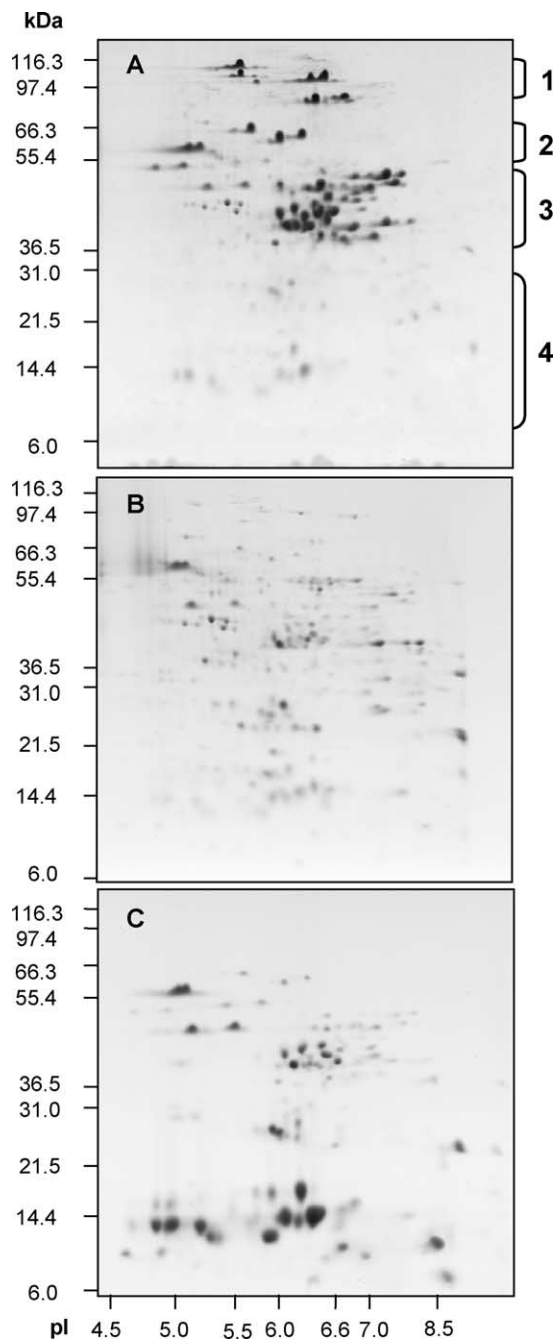


Fig. 2. 2-DE of wheat flour proteins separated by solubility in KCl and methanol. (A) KCl-insoluble/acetone-insoluble, gluten protein fraction, (B) KCl-soluble/methanol-insoluble, metabolic protein fraction, and (C) KCl-soluble/methanol-soluble, CM-like protein fraction. The gluten protein fraction contains high molecular mass-glutenin subunits (1),  $\omega$ -gliadins (2),  $\alpha$ - and  $\gamma$ -gliadins and low molecular mass-glutenin subunits (3), and albumins and globulins (4).

In a proteomic study of developing endosperm, we found that the majority of the proteins in this fraction were enzymes functional in biochemical processes ranging from carbohydrate metabolism and protein synthesis/assembly to storage and stress/defense (Vensel et al., unpublished

data). Only one gliadin, three  $\omega$ -gliadins, and five LMM-GS were identified in this fraction (DuPont et al., 2000). For these reasons, we have named this KCl-soluble/methanol-insoluble fraction the metabolic protein fraction.

The KCl-soluble/methanol-soluble fraction contained 5% of the total flour protein extracted; 2-DE resolved 180 proteins (Fig. 2C). The solubility of these proteins in methanol along with their mobilities indicated that they were CM-like proteins, i.e. low molecular mass storage proteins that are soluble in salt solution and in chloroform/methanol (Barber et al., 1986; Shewry et al., 1984). The most abundant proteins had a low molecular mass and were primarily  $\alpha$ -amylase inhibitors or  $\alpha$ -amylase/trypsin inhibitors;  $\alpha$ - and  $\gamma$ -gliadins were also prominent in this fraction (Wong et al., 2004).

By comparing Coomassie gels of the gluten protein fraction that had been destained in water for 3 h (Fig. 3A) or 24 h (Fig. 3B), we found that all of the proteins destained to some extent. However, the proteins in the  $\omega$ -gliadin region destained to a greater extent than the others (compare boxed proteins in Fig. 3A and B). A time course revealed that these proteins were intensely stained after destaining the gels for 1 h in water (Fig. 3C). However, high background staining reduced protein resolution, and an additional 2 h of destaining was required to decrease background staining adequately. While some reduction in staining of the  $\omega$ -gliadins was evident after 3 h in water, destaining was more conspicuous after 8 h and substantial after 24 h. These proteins did not stain again when the gels were re-stained with Coomassie blue, indicating they had diffused out of the gel (data not shown). This loss was prevented when gels destained for 3 h in water were placed in 20% ammonium sulfate; staining remained at similar levels whether gels were stored for 24 h or 5 d in ammonium sulfate.

In summary, we describe a simple and rapid method for separating flour proteins into fractions containing gluten, metabolic, and CM-like proteins. The method uses a relatively high concentration of SDS to solubilize the gluten proteins. Effective removal of the SDS using acetone allows these proteins to be further characterized by IEF as well as HPLC. An added advantage, especially for samples containing small amounts of protein, is that the use of methanol or acetone precipitation minimizes losses during sample manipulations. Our 2-DE method involves a number of steps that have been optimized for analysis of wheat endosperm proteins. During the course of these studies, we discovered that proteins in the  $\omega$ -gliadin region diffused out of the 2-D gels unless appropriate measures were taken during destaining and storage. This finding is an important consideration in studies that rely on protein quantification. Taken together, the fractionation and 2-DE methods will be useful for global studies of wheat endosperm proteins.

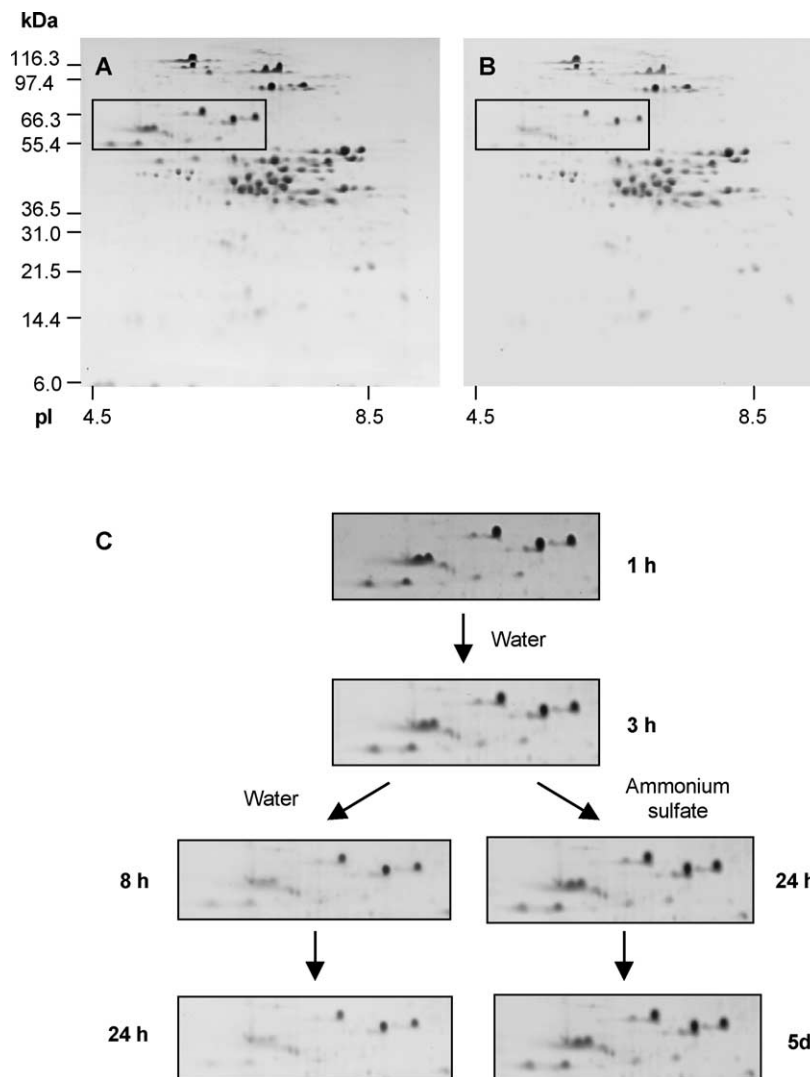


Fig. 3. Ammonium sulfate prevents destaining of the  $\omega$ -gliadins. 2-D gels stained with Coomassie blue were destained with water for 3 (A) or 24 (B) h. The box delineates proteins in the the  $\omega$ -gliadin region of the gel. These proteins were monitored in 2-D gels that were destained with water for 1, 3, 8, or 24 h or placed in 20% ammonium sulfate after 3 h for 24 h or 5 d (C).

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